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(54) Title: SUPPRESSION OF TUMOR CELL GROWTH BY SYNDECAN-1 ECTODOMAIN

(57) Abstract

Methods of reducing tumor growth by providing the ectodomain of syndecan are provided.

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SUPPRESSION OF TUMOR CELL GROWTH BY SYNDECAN-1 ECTODOMAIN

FIELD OF THE INVENTION

This invention is in the field of cancer biology and therapy. Specifically, the invention is to a method for slowing or normalizing the growth rate of a cell, especially a malignant cell, by providing efficacious amounts of the ectodomain part of syndecan-1 to such cell. The method of the invention facilitates and results in the normalization of the growth rate and differentiation state of malignant cells.

BACKGROUND OF THE INVENTION

Cellular differentiation is based on selective use of genetic information programmed by extracellular stimuli, which for example could include cellular interactions and binding of extracellular effector molecules by cell surface receptors. It is becoming more evident that cell surface proteoglycans play an important role in the regulation of cell behavior. Syndecans are cell surface proteoglycans, which have been shown to participate in both matrix recognition and growth factor binding and thus believed to participate in cell regulation. The sequences of human, mouse, rat and hamster syndecans are known. Syndecans have recently been reviewed (Jalkanen, *et al.*, in *Receptors for Extracellular Matrix*, J. MacDonald & R. Mecham, Editors, Academic Press, San Diego, pp. 1-37 (1991) and Bernfield, O., *et al., Annu. Rev. Cell Biol. 8*:365-393 (1992)).

Syndecan-1 is the best characterized cell surface proteoglycan (Saunders *et al., J. Cell Biol. 108*:1547-1556 (1989); Mali *et al.*, *J. Biol. Chem. 265*:6884-6889 (1990)). International patent application WO 90/12033 discloses the aminoacid sequence and corresponding cDNA sequence of mouse syndecan-1 molecule. A diagnostic method for detecting transformed cells by detecting changes is the syndecan expression in transformed cells is described in International Patent Application WO 92/13274 and WO 93/05167.

The enhancer element of the syndecan gene as well as a method of decreasing the growth of malignant cells by inducing the expression of syndecan within malignant cells is described in International Patent Application (PCT/FI93/00514)

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the sequence of human syndecan-1. Circles: possible GAG attachment sites; bold underline: transmembrane domain; light underlining: aataa polyadenylation signal.

Figure 2 is the sequence of mouse syndecan-1.

Figure 3 Schematized structure of the core proteins of wild type, tail-less and ecto transfection constructs. The wild type construct contains the full length mouse syndecan-1 ectodomain (Mali, M. *et al., J. Biol. Chem. 268*:24215 (1993)). The tail-less construct was generated using oligonucleotide-directed mutagenesis resulting a deletion mutant with single arginine residue in the cytoplasmic domain as described in the examples (Miettinen, H.M. *et al., J. Cell Sci.* in press (1994)). The ecto construct was also derived by oligonucleotide-directed mutagenesis as described in the examples, and has a stop codon in the protease sensitive site just adjacent to the cell surface. Vertical lines indicate putative GAG attachment sites and arrows the dibasic protease sensitive site.

Figure 4 Actin filament organization and immunofluorescence localization of syndecan-1 on the cell surface.

Figure 5 Amount of secreted ectodomain of syndecan-1 from the conditioned medium of Ecto cell clones (Ecto 15, 34, 2 and 23). Cells were cultured for two days in the presence of 10 nM testosterone and the ectodomain of syndecan-1 that accumulated in the medium was used. The culture medium was used directly. Samples were normalized for cell number and equivalent amounts slot-blotted on Hybond-N+ membrane. The ectodomain of syndecan-1 was detected by enhanced chemiluminescence method using 281-2 as described in the examples (Miettinen, H.M. *et al., J. Cell Sci.* in press (1994)). Quantitations were

done using computer image analysis system (Imaging Research Inc.). Means and SEMs of two parallel samples are presented.

Figure 6 Actin filament organization of Ecto cell clones. Ecto cells were cultured in the presence of 10 nM testosterone and actin filaments were visualized by rhodamine-conjugated phalloidin.

Figure 7 Soft-agar colony formation of Ecto cell clones. Cells were cultured 12 days in 0.33% soft-agar, DMEM+5% FCS with 10 nM testosterone as described earlier (Leppä, S. *et al., Proc. Natl. Acad. Sci. USA 89*:932 (1992)).

Figure 8 The effect of DEAE-isolated syndecan-1 ectodomain (examples) from the conditioned medium of Ecto 2 cells on growth of NMuMG and testosterone treated (10 nM) S115 cells (S115+). 1500 cells were transferred into 96-well culture plates and cells were cultured with DEAE-isolated syndecan-1 ectodomain until control (without syndecan-1 ectodomain) cells reached about 75-85% confluence (NMuMG cells four days, S115+ three days). Then cells were fixed with 2% paraformaldehyde, stained with 0.5 % crystal violet and washed with distilled water. Stained cells were suspended in 10% acetic acid and spectrophotometrically measured at 595 nm.

Figure 9 The effect of heparitinase treatment of DEAE-isolated syndecan-1 ectodomain on growth inhibition of S115+ cells. S115 + cells were cultured with 1 nM DEAE-isolated syndecan-1 from cultured medium of Ecto 2 cells and from the medium of NMuMG cells, or with the same preparations pretreated with heparatinase (Seikagaku Kogyo Co.) 1 hour at 37^{o}C.

Figure 10 The effect of immunopurified syndecan-1 ectodomain on growth of S115+ and NMuMG cells. DEAE-isolated syndecan-1 ectodomain was further purified with 281-2 immunoaffinity column (examples). S115+ and NMuMG cells were cultured with 1 nM immunoaffinity purified syndecan-1 ectodomain.

Figure 11 DEAE-isolated syndecan-1 ectodomain but not HS or CS GAGs inhibit growth of S115+ cells.

Figure 12 Growth inhibition of different cell line cells (CarB, MCF-7, S115+ with 10 nM testosterone, S115- without testosterone, NIH 3T3, NMuMG and HaCaT) by 1 nM DEAE-isolated syndecan-1 ectodomain (examples). Cell growth were analyzed in all panels similarly as in panel (A) and it was compared to the cells without treatments (% of control, y-axis). Means and SEMs from two parallel samples are presented.

Figure 13. Suppression of tumor growth in nude mice by syndecan-1 ectodomain.

SUMMARY OF THE INVENTION

The present invention is first directed to a pharmaceutically acceptable composition containing syndecan ectodomain.

The invention is further directed to a method for decreasing or normalizing tumor cell growth by providing such syndecan ectodomain protein to a tumor cell, in the cell's extracellular environment.

The methods of the inventions are useful with both malignant and non-malignant tumor cells, and are especially useful with tumors characterized by loss of syndecan-1, such as gliomas, myelomas, carcinomas, sarcomas, lymphomas or adenomas.

DEFINITIONS

In order to provide a clearer and more consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Cell growth. By "cell growth" is meant cell replication, or the rate of cell division, both controlled and uncontrolled. Therefore, cell growth is the rate of division and replication.

Malignant. By "malignant" is meant uncontrolled cell growth.

More Differentiated Phenotype. In stating that a cell has a "more differentiated phenotype" is meant that the cell possesses a phenotype

usually possessed by a certain cell type more differentiated than the cell. A phenotype can be defined by one or more phenotypic characteristics. For example, an epithelial cell shape is a more differentiated phenotype of a mesenchymal-like shape; therefore, in this example, the "more differentiated phenotype" is the epithelial cell morphology, rather than a mesenchymal-like shape. A terminally differentiated mesenchymal cell is a "more differentiated phenotype" than a condensing mesenchymal cell. The state of the actin-containing cytoskeleton can also be used; disorganized actin filaments are indicators of a less differentiated phelotype than organized filaments.

Efficacious Amount. An "efficacious amount" of an agent is an amount of such agent that is sufficient to bring about a desired result, especially upon administration of such agent to an animal or human. An efficacious amount of syndecan-1 ecdodomain in the compositions and methods of the invention is the amount sufficient to reduce tumor cell growth, preferably to normal growth rates for the specific cell types.

Administration. The term "administration" is meant to include introduction of the syndecan ectodomain according to the invention into an animal or human by any appropriate means known to the medical art, including, but not limited to, injection, oral, enteral, transdermal and parenteral (e.g., intravenous) administration.

Exposure to syndecan ectodomain. By "exposing" a cell to syndecan ectodomain in the compositions of the invention is meant that the external milieu of the cell is provided with amounts of syndecan ectodomain that are efficacious in promoting the desired effect, generally a lowered growth rate of a tumor cell.

Pharmaceutically Acceptable Salt. The term "pharmaceutically acceptable salt" is intended to include salts of the syndecan ectodomain of the invention. Such salts can be formed from pharmaceutically acceptable acids or bases, such as, for example, acids such as sulfuric, hydrochloric, nitric, phosphoric, etc., or bases such as alkali or alkaline earth metal hydroxides, ammonium hydroxides, alkyl ammonium hydroxides, etc.

Pharmaceutically Acceptable Composition. The term "pharmaceutically acceptable composition" is intended to include solvents, carriers, diluents, and the like, which are utilized as additives or vehicles to preparations of the syndecan ectodomain of the invention so as to provide a carrier or adjuvant for the administration of such compounds to patients (human or animal) in need of the same. Such additives can perform certain functions, such as, for example, provide the proper ionic conditions for administration, stabilize the syndecan ectodomain against inactivation or degradation, and/or increase the half-life of the syndecan ectodomain. A pharmaceutically acceptable composition is medically compatible with the host to which it is being administered.

Treatment. The term "treatment" or "treating" is intended to include the administration of the pharmaceutically acceptable compositions of the invention comprising efficacious amounts of syndecan ectodomain of the invention to a patient for purposes which may include prophylaxis, amelioration, prevention or cure of a medical disorder, including the suppression of tumor growth.

Substantially Free of Natural Contaminants. A material is said to be "substantially free of natural contaminants" if it has been substantially purified from materials with which it is normally and naturally found before such purification and those contaminants normally and naturally found with the substance *in vivo* or *in vitro* are substantially absent from the final preparation of the material. When administered to a subject in need of treatment, the syndecan ectodomain of the invention is substantially free of natural contaminants which associate with the syndecan ectodomain either *in vivo* (in the host from which the ectodomain was isolated), or *in vitro* (as a result of a chemical synthesis). By "substantially absent" is meant that such contaminants are either completely absent or are present at such low concentrations that their presence (1) does not interfere with the desired therapeutic effect of the active agent (herein the abiltiy of the syndecan ectodomain to inhibit tumor growth) in the therapeutically acceptable composition when such composition is administered to a patient in need of same and (2) does not harm the patient as the result of the administration of such composition.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention is the discovery that the ectodomains of the syndecans possess certain biological functions and are capable of providing such functions to cells when presented to the external surface of a cell other than the cell that synthesized such syndecan ectodomain. Syndecans are membrane bound proteins. It was surprisingly found that extracellularly-provided syndecan ectodomain, by itself, is sufficient to restore a more differentiated morphology to tumor cells and to suppress the growth of malignant cells. The invention herein is exemplified with syndecan-1.

All syndecans contain a cytoplasmic domain, a transmembrane domain and an extracellular domain. The extracellular domain is the ectodomain. As discussed by Jalkanen, *et al.*, in *Receptors for Extracellular Matrix*, J. MacDonald & R. Mecham, Editors, Academic Press, San Diego, pp. 1-37 (1991)), the syndecans show highly conserved homologous sequences at three separate regions of their ectodomains. A dibasic sequence is immediately adjacent to the N-terminal end of the hydrophobic transmembrane domain, suggesting that it is located next to the outer leaflet of the plasma membrane, and may serve as a protease-susceptible site, which enables the ectodomain to be cleaved intact from the cell surface.

The core protein of human syndecan-1 contains 310 amino acid residues. There is a high degree of structural and functional homology between mouse and human syndecan-1. Human syndecan-1 has an identical size, charge, buoyant density and GAG composition to that of mouse syndecan-1. Human syndecan-1 ectodomain, like that of the mouse, binds to type I collagen fibrils and fibronectin but not to laminin or virtronectin.

The sequence of human syndecan-1 is known and it has been cloned (Mali *et al., J. Biol. Chem. 265*:6884-6889 (1990)). When numbered according to Figure 2 in Mali *et al., J. Biol. Chem. 265*:6884-6889 (1990), amino acids 1 to 251 are the ectodomain of human syndecan-1 (with the secretion-signal attached), the hydrophobic membrane-spanning domain contains the next 25 amino acid residues

(amino acids 252-276), and the cytoplasmic domain contains the last 34 amino acid residues (amino acids 277-310).

The signal peptide sequence is the first 17 amino acids of the ectodomain. Although useful to promote secretion of syndecan-1 from a cell synthesizing the same, the secretion signal is not necessary for the tumor growth suppression or differentiation functions of the ectodomain of the invention.

Therefore, the sequence of the ectodomain of the invention included those fragments of syndecan amino acid residues 1-251 that retain the GAG attachments sites and desired function of the ectodomain, such as, for example, ectodomains having amino acids 1-251 (with secretion signal and cleaved at the RK site), 18-251 (minus secretion signal but cleaved at the RK site), 1-231 (with secretion signal but cleaved at the RR site) and 18-251 (minus secretion signal but cleaved at the RR site). An ectodomain having a carboxy terminal at a site anywhere between amino acid residues 231-251, or a secretion signal fragment of less than amino acids 1-17 is also useful since those embodiments would be expected to retain the biological properties of the ectodomain.

Although the human and mouse ectodomains are only 70% identical at the amino acid level, all putative glycosaminoglycan (GAG) attachments sites are identical between the mouse and human sequences. The five possible glycosaminoglycan attachment sites of human syndecan ectodomain are at positions 37, 45, 47, 206 and 216. Two of these sites belong to the consensus sequence SGXG and three others to (E/D)GSG(E/D). Also identical between mouse and human syndecan are the single site for N-glycosylation and the proteinase-sensitive dibasic RK site adjacent to the extracellular face of the transmembrane domain. Human syndecan also contains a second dibasic RR sequence just 18 residues apart from the RK sequence. Proteolytic cleavage at this site would also release an ectodomain of the invention that contained all GAG sites intact.

The transmembrane domains of human and mouse syndecan-1 are 96% identical (the only change in human syndecan is an alteration

of an alanine to a glycine) and the cytoplasmic domains are 100% identical in mouse and human syndecan.

Syndecan ectodomain, such as human syndecan ectodomain, can be produced by recombinant techniques in any desired host. However, it is preferable, but not necessary, to utilize a host that is of a similar cell type to that of the tumor, so as to provide as similar GAG composition as possible, to that of the cell in its non-tumor state. Many deposited cell lines that are human tissue specific or characteristic of different cell types are available.

For example, the mouse syndecan-1 clones of the invention were constructed using liposome transfection and geneticin to subsequent select stably transfected cells clones. S115 cell line clones (see Fig. 3) expressing either the wild type mouse syndecan-1 (wild type), a deletion mutant with a single arginine residue in the cytoplasmic domain only (tail-less) or the plain ectodomain of syndecan-1 (ecto). Wild type syndecan-1 and cytoplasmic deletion mutant (tail-less) were cloned into EcoRI site of the pBGS eucaryotic expression vector. The ectodomain construct was cloned into pMAMneo vector, in order to obtain efficient expression levels also in the presence of hormone since the MMT LTR promoter is induced by the same steroid hormone as the cells. It is not necessary to use this vector as many such expression vectors are known in the art. Syndecan-1 expression at the cell surfaces was detected using a monoclonal antibody, exemplified using previously described mAb 281-2, that recognizes the ectodomain of mouse syndecan-1 core protein, and actin filaments were visualized using rhodamine-conjugated phalloidin, as an indication of the differentiation state and growth state of the cell.

Without testosterone, S115 cells exhibit organized actin filaments typical to these cells when epithelioidal. In the presence of testosterone, actin was disorganized and globular, and the cell surface expression of syndecan-1 was also suppressed. Wild type and Tail-less clones expressing syndecan-1 at the cell surfaces restored actin filament organization in spite of the testosterone treatment. Because transfection of Tail-less mutant also induced similar changes as the Wild type syndecan-1, S115 cells were transfected with the plain ectodomain and

more than 50 independent clones secreting different levels of the ectodomain into the culture medium were produced. The cell surfaces of these cells stained only faintly for syndecan-1 but still these cells revealed well organized actin filaments and an epitheloid morphology. These results indicate that ectodomain of syndecan-1 is sufficient enough to restore epithelioid morphology of testosterone treated S115 cells to that of the more differentiated phenotype and is a useful anti-cancer drug.

In non-tumor cells, syndecan is expressed in epithelial cells, mesenchymal cells, pre-B cells and plasma cells, but not by B cells. Syndecan is also expressed in tissues that contains cells of this type, including human brain tissue. Therefore the methods of the invention are especially useful against tumors of the epithelial, mesenchymal, pre-B and plasma cells. Most especially, the methods of the invention are useful in slowing the growth of steroid responsive tumors, especially estrogen or androgen responsive tumors (tumors that grow better in the presence of steroids, estrogn, or androgens as indicated) including breast cell tumors, endometrium cell tumors, and tumors of the prostate cells.

For treatment of humans and animals, syndecan-1 ectodomain is administered in a pharmaceutically acceptable solution at levels sufficient to restore the normal growth state of tumor, or malignant cells, as evidenced by a slower growth rate. The syndecan-containing pharmaceutically acceptable solution can be administered in any form that effects prophylactic, palliative, preventative or regressive tumor growth.

The amount of the syndecan ectodomain-1 compositions of the invention that is administered to the patient, and the duration of such administration, can be determined by monitoring tumor growth in the patient during the course of the administration, and adjusted according to the response of the patient. The syndecan ectodomain of the invention is preferably provided to the target tumor cell at extracellular concentrations about 0.7 nM-1 nM (see Figure 11), but any concentration sufficient to decrease growth of the tumor may be used. The ectodomain can be provided either locally (as with a concentrated

delivery right to the targetted organ) or systemically (such as delivery through the blood stream). The dose of syndecan given to the patient (either human or animal) will therefore take into account the volume (such as blood volume) into which the ectodomain is being administered, and the type of tumor that is being targeted. For example, if a continuous exposure to the syndecan ectodomain is necessary, then more frequent dosages will be required than if only a transient exposure of the tumor to the syndecan ectodomain is necessary. For example, a 1 nM amount of syndecan ectodomain having amino acids 1-251 corresponds to 0.2 mg/L (200 μg/L), either in the blood or locally concentrated at the site of action. Typical systemic doses of syndecan ectodomain useful in the methods of the invention for treatment of humans or animals include amounts that provide a final blood concentration of most preferably 0.2 mg syndecan ectodomain per liter blood. Blood volume in humans is 6% of the body weight, hence a 70 Kg person has about 4.2 liters of blood. However, because the effects of the syndecan ectodomain are presumably local (e.g. acting at a specific cell membrane), sequestered or kinetically determined, the theoretically minimum dose can be adjusted upward in order to achieve favorable therapeutic effects.

Syndecan ectodomain may be administered by any route that delivers efficacious levels of the drug to the desired active site, for example, by injection. For parenteral administration, preparations containing the syndecan ectodomain may be provided to a patient in need of such treatment in combination with pharmaceutically acceptable sterile aqueous or non-aqueous solvents, suspensions and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil, fish oil, and injectable organic esters. Aqueous carriers include water, water-alcohol solutions, emulsions or suspensions, including saline and buffered medical parenteral vehicles including saline and buffered medical parenteral vehicles including sodium chloride solution, Ringer's dextrose solution, dextrose plus sodium chloride solution, Ringer's solution containing lactose, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based upon Ringer's dextrose and the like.

The syndecan ectodomain containing medicament (the pharmaceutically acceptable solution containing the therapeutically active syndecan-1 ectodomain) can be administered by means of catheters or pumps, especially when it is desired to deliver the ectodomain at localized high concentrations. The syndecan-1 ectodomain-containing medicament can be administered subcutaneously or directly into soft tissue by means of implantaion devices inert to body fluids. Such devices and implantation systems are known in the art. A ceramic sytem for delivery proteins is described, for exmaple, in WO 92/00109.

The syndecan-1 ectodomain containing medicament can be administered by providing such molecule as a part of a chimeric molecule (or complex) which is designed to target specific organs, for example, as part of an antibody that recognizes determinants on the target tissue or organ or cell, in its tumor or nontumor state.

The pharmaceutically acceptable solution contining the sydnecan-1 ectodomain can be administered topically. Although syndecan-1 ectodomain can be administered to a patient in a regime that includes other cancer fighting drugs, optimal administration of the syndecan-containing compositions of the invention are especially useful in this regard.

Topical adminsitration is preferably accomplished in one of two ways. First, the therapeutically active syndecan ectodomain can be mixed with suitable pharmaceutially acceptable carriers and (optionally), penetration enhancers to assist in the delivery of the active agent across the skin, to form ointments, emulsions, lotions, solutions, creams, gels or the like, and the preparation itself is then applied to a certain area of skin. Alternatively, the therapeutically active syndecan ectodomain can be incorporated into a patch or transdermal delivery system according to known technology for the preparation of such patches and delivery systems.

Administration in a sustained-release form is more convenient for the patient when repeated injections for prolonged periods of time are needed, or when continuous exposure of the tumor cell to the

ectodomain is desired. In intravenous dosage forms the compositions of the present invention have a sufficiently rapid onset of action to be useful in the acute management of tumor growth.

Administration may be localized directly to the cell if the cell is associated with a tissue or bodily organ, or administration can be systemic, in a medium in which the cell is found, such as the blood or cerebrospinal fluid. Systemic administration throughout the patient's body, for example, by administration to the bloodstream, facilitates treating patients for whom tumor cells may be at more than one site in the body.

Providing syndecan ectodomain as the product of a syndecan ectodomain expression construct that secretes ectodomain in efficacious amounts is also considered "administration." For example, administration across the blood brain barrier can be achieved by utilizing known viral vector systems to deliver syndecan ectodomain DNA in a manner that expresses ectodomain and secretes it to the extracellular environment, such as, for example, in the retroviral systems described in WO 93/03743, WO90/09441, Breakefield, X.A. *et al., The New Biologist 3*:203-218 (1991) and Huang, Q. *et al., Exp. Neurol. 115*:303-316 (1992).

The pharmaceutically acceptable composition of the invention, containing the syndecan-1 ectodomain can be manufactured in a manner which is in itself known, for example, by means of conventional mixing, dissolving, lyophilizing or similar processes. The compositions of the present invention that provide the syndecan-1 ectodomain find utility in their ability to slow or prevent tumor growth or tumor reappearance, and in their ability to alter the phenotype of the cell to that a more differentiated state, in both human and animal patients. The syndecan-1 ectodomain compositions of the invention utilize the body's own mechanisms for promoting differentiation of specific cell types to its maximum potential.

The compositions and methods of the invention are not meant to be limited to syndecan-1. Syndecan-1, syndecan-2, syndecan-3 and syndecan-4 are known to contain similar domain structures. It is known

that differentiation of certain cell types is associated with the loss of syndecan-1 but with the appearance of another member of the syndecan family (Bernfield, O., *et al., Annu. Rev. Cell Biol. 8*:365-393 (1992)). For example, when bronchial epithelia form buds, lung mesenchyme loses syndecan-1 but acquires syndecan-2. In tumors from cell types that lose syndecan-1 upon differentiation but express a different syndecan, utilization of the ectodomain from the syndecan that is expressed in the differentiated state would be indicated.

The examples below are for illustrative purposes only and are not deemed to limit the scope of the invention.

EXAMPLES

The following examples are intended to illustrate, but not to limit the invention.

EXAMPLE 1

Deletion mutant syndecan constructs

Using liposome transfection and subsequent selection of stably transfected cells clones by geneticin as described by Leppä et al., *Proc. Natl. Acad. Sci. U.S.A. 89*: 932 (1992), S115 cell line clones (see Fig. 3) were produced that expressed either the wild type mouse syndecan-1 (Wild type), a deletion mutant with a single arginine residue in the cytoplasmic domain only (Tail-less) or only the ectodomain of syndecan-1 (Ecto 2; see Fig. 3). These three forms and the hosts were constructed as follows.

The full-length mouse syndecan-1 cDNA, as described in Mali *et al., J. Biol. Chem. 268*:24215-24222 (1993) was cloned into the EcoRI site of Bluescript SK+ (Promega).

1) The EcoRI insert of the Bluescript construct was cloned into the EcoRI site of the pBGS vector (Mali *et al., J. Biol. Chem. 268*:24215-24222 (1993)) and the orientation was confirmed. This construct was designated "Wild-type."

2) A mutagenic 25-base oligonucleotide having the sequence: 5'G CTG TAC CGC TAG CAG AAG AAG GAC-3' [SEQ ID No. 1], containing a stop codon and a NheI restriction site (underlined) was used to convert the codon for the second amino acid (methionine) of the cytoplasmic domain following the transmembrane domain to a stop codon. The mutation was confirmed by restriction digestion and dideoxy sequencing. The EcoRI insert of the Bluescript construct was cloned into the EcoRI site of an amplifiable pBGS vector (Mali *et al., J. Biol. Chem. 268*:24215-24222 (1993)). This mutant syndecan-1 containing one amino acid (arginine) in its putative cytoplasmic domain was designated "Tail-less."

A mutagenic 33-base oligonucleotide 5′-GACACCTCCCAGTACTCACTTCCTGTCCAAAAG-3′ [SEQ ID No. 2] containing a stop codon (bolded) and a ScaI site (underlined) was used to convert the first codon (E) after the dibasic protease sensitive site of the ectodomain to a stop codon. The mutation was confirmed by restriction digestion and dideoxy sequencing. This was the Bluescript-ecto construct. The EcoRI insert of the Bluescript-ecto construct was cloned into the EcoRI site of pJC119R vector (Miettinen *et al., J. Cell Sci. 107*: in press, (1994)). XhoI digested ecto insert from pJC119R-ecto construct was ligated into XhoI site of pMAMneo eucaryotic transfection vector, available from Clontech, Palo Alto (Leppä et al., *Proc. Natl. Acad. Sci. U.S.A.* 89, 932 (1992)), and the orientation was confirmed by restriction digestions.

EXAMPLE 2

Expression of mutant syndecan-1 normalizes malignant growth in S115 cells

Wild type syndecan-1 and cytoplasmic deletion mutant (Tail-less) were cloned into the EcoRI site of the pBGS eucaryotic expression vector (Mali et al., *J. Biol. Chem. 268*: 24215 (1993), but the ectodomain construct was cloned into pMAMneo vector, in order to obtain efficient expression levels also in the presence of hormone (personal communication, S. Ala-Uoti, Turku Centre for Biotechnology).The pBGS system is not repressed by testosterone. Syndecan-1 expression at the cell surfaces was detected using mAb 281-2 (Jalkanen et al., *J. Cell Biol.*

101: 976 (1985)) that recognizes the ectodomain of mouse syndecan-1 core protein, and actin filaments were visualized using rhodamine-conjugated phalloidin.

Cells (S115+, wild type, tail-less and Ecto 2) were cultured four days on coverslips in DMEM-5% FCS-1mM Na-pyruvate with 10 nM testosterone, except S115- cells which were cultured without testosterone in DMEM-4% DCC-FCS (Dextran-Coated-Charcoal treatment eliminates endogenous steroids from serum) with 1mM Na-pyruvate. Cells were fixed with 0.1% Triton-X-100, 2% paraformaldehyde and incubated with rhodamine-conjugated phalloidin (Sigma). Cell surface syndecan-1 expression was visualised by incubating living cells for 1 hour on ice with rat mAb 281-2 (recognizes mouse syndecan-1 ectodomain); they were then fixed with 2% paraformaldehyde and bound mAb 281-2 was visualized using FITC-conjugated rabbit anti-rat IgG.

Without testosterone S115 cells exhibited organized actin filaments typical to these cells when epithelioidal. In the presence of hormone actin was disorganized and globular, and the cell surface expression of syndecan-1 was also suppressed as shown earlier by Leppä et al., *Cell Reg.* 2,1 (1991), Fig. 4.

Wild type and Tail-less clones expressing syndecan-1 at the cell surfaces restored actin filament organization in spite of the testosterone treatment, Fig. 4.

EXAMPLE 3

Effect of secreted syndecan-1 ectodomain on cultured S115 cells

Because transfection of the Tail-less mutant induced changes similar to those of the wild type syndecan-1, S115 cells were transfected with the ectodomain. More than 50 independent clones secreting different levels of the ectodomain into the culture medium (see Figure 5, 6 and 7) were produced. The cell surfaces of these cells stained only faintly for syndecan-1 but still these cells revealed well organized actin filaments and an epitheloid morphology (Fig. 4). These results

suggested that ectodomain of syndecan-1 is sufficient enough to restore epithelioid morphology of testosterone treated S115 cells.

To analyze in detail Ecto clones, amounts of secreted syndecan-1 ectodomain from the culture media were measured by enhanced chemiluminescence method using mAb 281-2 against ectodomain of syndecan-1 core protein. Two separate stably transfected cell clones secreting high amounts of syndecan-1 into the culture medium (Ecto 2 and Ecto 23) and two cell clones with low expression (Ecto 15 and Ecto 34) were selected for further analysis (Fig. 5).

A clear correlation between syndecan-1 ectodomain expression and re-organization of actin filaments was detected in the presence of 10 nM testosterone: Ecto 15 and Ecto 34 with low syndecan-1 expression had disorganized, mainly globular actin, but Ecto 2 and Ecto 23 clones expressing syndecan-1 ectodomain exhibited epithelioid morphology with organized actin filament bundles (Fig. 6). Enhanced expression of intact syndecan-1 has been shown previously to suppress tumor growth of testosterone-treated S115 cells (Leppä et al, *supra*), and now also Ecto 2 and Ecto 23 clones with high syndecan-1 ectodomain expression restricted their growth in soft-agar. The low syndecan-1 ectodomain expressing clones Ecto 15 and Ecto 34 clones, however, demonstrated soft-agar growth typical to parental S115 cells (Fig. 7). Soft agar experiment indicated that in addition to morphology, syndecan-1 ectodomain expression is sufficient to restrict also the tumorigenic growth of S115 cells.

EXAMPLE 4

Isolation and purification of syndecan ectodomain from Ecto cell cultures

Because syndecan-1 ectodomain seemed to be responsible for the suppression of the malignant growth behavior of androgen treated S115 cells, we collected conditioned medium from Ecto cell cultures for ectodomain isolation. Conditioned cell culture medium was denatured with 2M urea and boiling, before loading to DEAE-sephacel column, 50 mM Na-acetate (pH=4.5) was added and medium was chilled to +4°C. The column was washed with 0.2 M NaCl, 2 M urea, 50 mM Na-acetate (pH=4.5), and the bound material was eluted using 1 M NaCl, 2 M urea,

50 mM Na-acetate (pH=4.5). Fractions containing syndecan-1 ectodomain was dialyzed against phosphate buffered saline (PBS) at 4°C. Amount of syndecan-1 ectodomain in fractions was estimated by slot-blotting and subsequent enhanced chemiluminescence method using mAb 281-2 (Example 2 and Miettinen, H.M. *et al., J. Cell Sci.* in press (1994)) and comparing the amount to the known syndecan-1 standard.

Ectodomain of syndecan-1 from cultured medium of Ecto cells was biochemically similar to the syndecan-1 ectodomain isolated from normal murine mammary epithelial cells (NMuMG). After isolation, the syndecan-1 content of the preparate was measured and the preparate tested on hormone-treated S115 cells. As shown in Fig. 8, concentrations of the DEAE-isolated syndecan-1 ectodomain as low as 1 nM suppressed the growth of testosterone treated S115 cells (Fig. 8). The same concentration only slightly inhibited the growth of NMuMG cells, which served as normal epithelial cells (Fig. 8). Syndecan-1 ectodomain was also isolated from the culture medium of NMuMG cells, and also with this preparate, a 1 nM concentration inhibited growth of hormone-treated S115 cells (Fig. 9). Treatment of the DEAE-isolated ectodomain with heparitinase totally abolished the growth inhibitory activity of these preparates (Fig. 9), suggesting that the core protein of syndecan-1 as such was not involved.

The DEAE-isolated syndecan-1 ectodomain was further purified using a mAb 281-2 immunoaffinity column: DEAE-isolated syndecan-1 ectodomain in PBS was loaded onto a mAb 281-2-Sepharose CL-4B immunoaffinity column as described in Jalkanen et al., *J. Cell Biol. 105*: 3087 (1987), and the bound material was eluted with 50 mM triethylamine (pH=11.5). Fractions containing syndecan-1 ectodomain were dialyzed against distilled water and subsequently lyophilized. After that syndecan-1 ectodomain suspended in DMEM (Gibco) and the amount was estimated, as described above. Again, at 1 nM concentrations of this immunoaffinity purified syndecan-1 ectodomain, growth inhibition of testosterone-treated S115 cells was observed and only a mild effect was evident with NMuMG cells (Fig. 10). On the other hand, heparin sulfate (HS) or chondroitin sulfate (CS) glycosaminoglycan chains alone did not suppressed S115 cell growth,

even if used at thousand-fold higher concentrations than syndecan-1 ectodomain (Fig. 11).

EXAMPLE 5

Effect of isolated syndecan-1 ectodomain on cultured cell lines

The inhibitory effect of the isolated syndecan-1 ectodomain was also tested on several other cell lines. These included poorly differentiated squamous cell carcinoma cells (CarB), human mammary tumor cells (MCF-7; ATCC HTB 22), S115 cells with (S115+) and without hormone (S115-), NIH 3T3 fibroblasts (ATCC CRL 1658), normal mammary epithelial cells (NMuMG; ATCC CRL 1636), and human keratinocyte cells (HaCaT; Fig. 12).

Cells were cultured and analyzed as described in Fig. 8 in the following mediums during the indicated periods of time: CarB cells (M. Quintanilla, K. Brown, M. Ramsden, A. Balmain, Nature 322, 78 (1991)) were cultured in HAM-F12-10% FCS for four days; MCF-7 cells in DMEM-5% FCS supplemented with 10 nM estradiol (E_2) and 10 µg/ml insulin for 4 days; S115+ and S115- cells were cultured as in Fig. 3 for three days; NIH 3T3 cells in DMEM-5% FCS for 4 days, NMuMG and HaCaT cells in 10% FCS-DMEM for 4 days. Because S115- cells have much slower growth rate than S115+ cells, 3000 S115- cells (other cell lines 1500 cells) were proportionally added to the well, so as to provide comparable results with the S115+ cells. Therefore, for S115- cells, 3000 cell were transferred to the plate as opposed to 1,500 cells for the other samples.

Those cell lines which form tumors (CarB, MCF-7, S115+), revealed strong growth suppression when exposed to syndecan-1 ectodomain at a 1 nM concentration (Fig. 12). In constrast, only moderate or no inhibition was observed with rest of the tested cell lines (S115-, NIH 3T3, NMuMG, HaCaT; Fig. 12), which all are all regarded as non-tumorigenic. Hormone exposure doubles the growth rate of S115 cells (Leppä et al., *supra*) but if syndecan-1 ectodomain is included in the cultures, the growth of S115 cells without androgen was 5.4 times higher than the growth of the same S115 cells with testosterone (Fig.

12). This was due to inhibition of the "malignant" behaving S115+ cells and undisturbed growth of epitheloid S115- cells.

EXAMPLE 6

Suppression of tumor in vivo-growth by syndecan-1 ectodomain

The ecto construct was made as described in earlier examples using the full length mouse syndecan-1 cDNA cloned in the Bluescript SK+ vector (10) and a mutagenic 33-base oligonucleotide 5'-GACACCTCCCAGTACTCACTTCCTGTCCAAAAG-3' [SEQ ID No.: 2] containing a stop codon (Bold) and a ScaI site (CAGTAC) to convert the first amino acid (E) after the dibasic protease-sensitive site of the ectodomain to a stop codon. The mutation was selected by restriction digestion and confirmed by dideoxy sequencing. Wild type syndecan-1 and the cytoplasmic deletion mutant were cloned into the EcoRI site of the pBGS eukaryotic expression vector (Mali et al., J. Biol. Chem. (1993) 268, 24215-24222). The ecto mutant was ligated into the XhoI site of the pMAMneo eucaryotic transfection vector (Leppä et al., PNAS (1992) 89, 932-936) because we knew that pMAMneo transfected S115 cells work well in a bioreactory system (personal communication, Sari Ala-Uotila, Turku Centre for Biotechnology). S115 cells were transfected using liposome transfection and subsequent selection with Geneticin as described earlier (Leppä et al., PNAS (1992) 89, 932-936).

S115 cells and transfection cell clones were cultured in DMEM-5% FBS-1 mM Na-pyruvate with 10 nM testosterone, except for S115- cells which were cultured without testosterone in DMEM-4% DCC-FBS (Dextran-Coated-Charcoal treated-fetal bovine serum, eliminates endogenous steroids from serum) with 1mM Na-pyruvate.

For tumor growth subconfluent cultures were detached with trypsin, washed with DMEM and counted with Coulter Counter (Coulter Electronics). Cells were resuspended in DMEM at a density of 5 x 10^7/ml and kept on ice until injection. Athymic male nude mice (nu/nu-BALB/cABom) between 6-8 week old (Bomholtgård, Rye, Denmark) were injected subcutaneously with 0.2 ml of the cell suspension. A silastic testosterone capsule was simultaneously implanted. Nude mice

were observed regularly for tumor development and the size of the tumors was measured at intervals in two perpendicular dimensions. When the animals were sacrificed, the lung and liver was evaluated for the possible appearance of metastases. The tumor sizes were measured on days 6, 11 and 15 after injcetion and are blotted as means of five individual tumors in Figure 13. The ectodomain transfected cells formed only acute inflammatory reaction and did not reveal tumor growth, opposite to wild type cells, which formed rapidly growing tumors. This experiment shows the efficacy of syndecan-1 ectodomain as a tumor suppressive agent *in vivo.*

All references cited herein are fully incorporated herein by reference. Having now fully described the invention, it will be understood by those with skill in the art that the scope may be performed within a wide and equivalent range of conditions, parameters and the like, without affecting the spirit or scope of the invention or any embodiment thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: SUPPRESSION OF TUMOR CELL GROWTH BY SYNDECAN-1 ECTODOMAIN

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: WO TO BE ASSIGNED
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/258862
(B) FILING DATE: 13-JUN-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCTGTACCGC TAGCAGAAGA AGGAC

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GACACCTCCC AGTACTCACT TCCTGTCCAA AAG

WHAT IS CLAIMED IS:

1. A method of decreasing the growth of a tumor cell wherein said method comprises providing efficacious levels of syndecan ectodomain to the extracellular environment of said cell.

2. The method of claim 1, wherein said cell is selected from the group consisting of epithelial cells, mesenchymal cells, pre-B cells and plasma cells.

3. The method of claim 2, wherein said cell is selected from the group consisting of a breast cell, an endometrium cell and a prostate cell.

4. The method of claim 3, wherein said cell is steroid-responsive.

5. The method of claim 4, wherein said steroid is estrogen or androgen.

6. The method of claim 1, wherein said cell is a human cell.

7. The method of claim 6, wherein said syndecan ectodomain is that of the human syndecan-1 of Figure 1.

8. The method of claim 7, wherein said ectodomain comprises amino acids 18-231 of figure 1 but not the transmembrane or cytoplasmic domain as shown in amino acids 252-310 of Figure 1.

9. The method of claim 8, wherein said ectodomain comprises amino acids 18-251 of Figure 1.

10. A method for treating a patient in need of treatment to reduce or suppress the growth of a tumor in said patient, wherein said method comprises administering to said patient, a composition that comprises efficacious levels of syndecan ectodomain to the extracellular environment of said cell.

11. The method of claim 10, wherein said cell is selected from the group consisting of epithelial cells, mesenchymal cells, pre-B cells and plasma cells.

12. The method of claim 11, wherein said cell is selected from the group consisting of a breast cell, an endometrium cell and a prostate cell.

13. The method of claim 12, wherein said cell is steroid-responsive.

14. The method of claim 13, wherein said steroid is estrogen or androgen.

15. The method of claim 10, wherein said cell is a human cell.

16. The method of claim 15, wherein said syndecan ectodomain is that of the human syndecan of Figure 1.

17. The method of claim 16, wherein said ectodomain comprises amino acids 18-231 of Figure 1 but not the transmembrane or cytoplasmic domain as shown in amino acids 252-310 of Figure 1.

18. The method of claim 17, wherein said ectodomain comprises amino acids 18-251 of Figure 1.

19. A pharmaceutically acceptable composition for administration to a patient, said composition comprising a protein having a domain consisting of the syndecan ectodomain.

20. The pharmaceutically acceptable composition of claim 19, wherein said syndecan is human and having the sequence shown in Figure 1.

21. The pharmaceutically acceptable composition of claim 20, wherein said ectodomain comprises amino acids 18-231 of Figure 1 but

not the transmembrane or cytoplasmic domain as shown in amino acids 252-310 of Figure 1.

22. The pharmaceutically acceptable composition of claim 20, wherein said ectodomain comprises amino acids 18-251 of Figure 1.

```
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FIGURE 1

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                                                                                   Me
```

Figure 2 (Sheet 1 of 6)

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Figure 2 Cont. (Sheet 2 of 6)

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Figure 2 (cont) Sheet 3 of 6

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Figure 2 (Cont.) sheet 4 of 6

```
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Figure 2 (cont) (Sheet 5 of 6)

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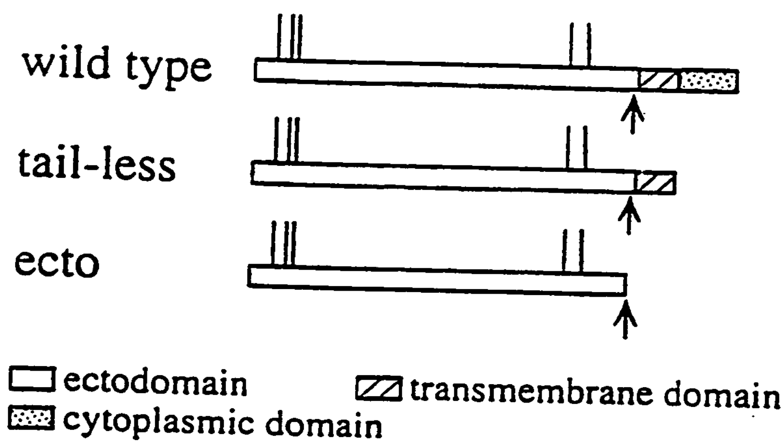
Figure 2 (Cont.) (Sheet 6 of 6)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ectodomain
transmembrane domain
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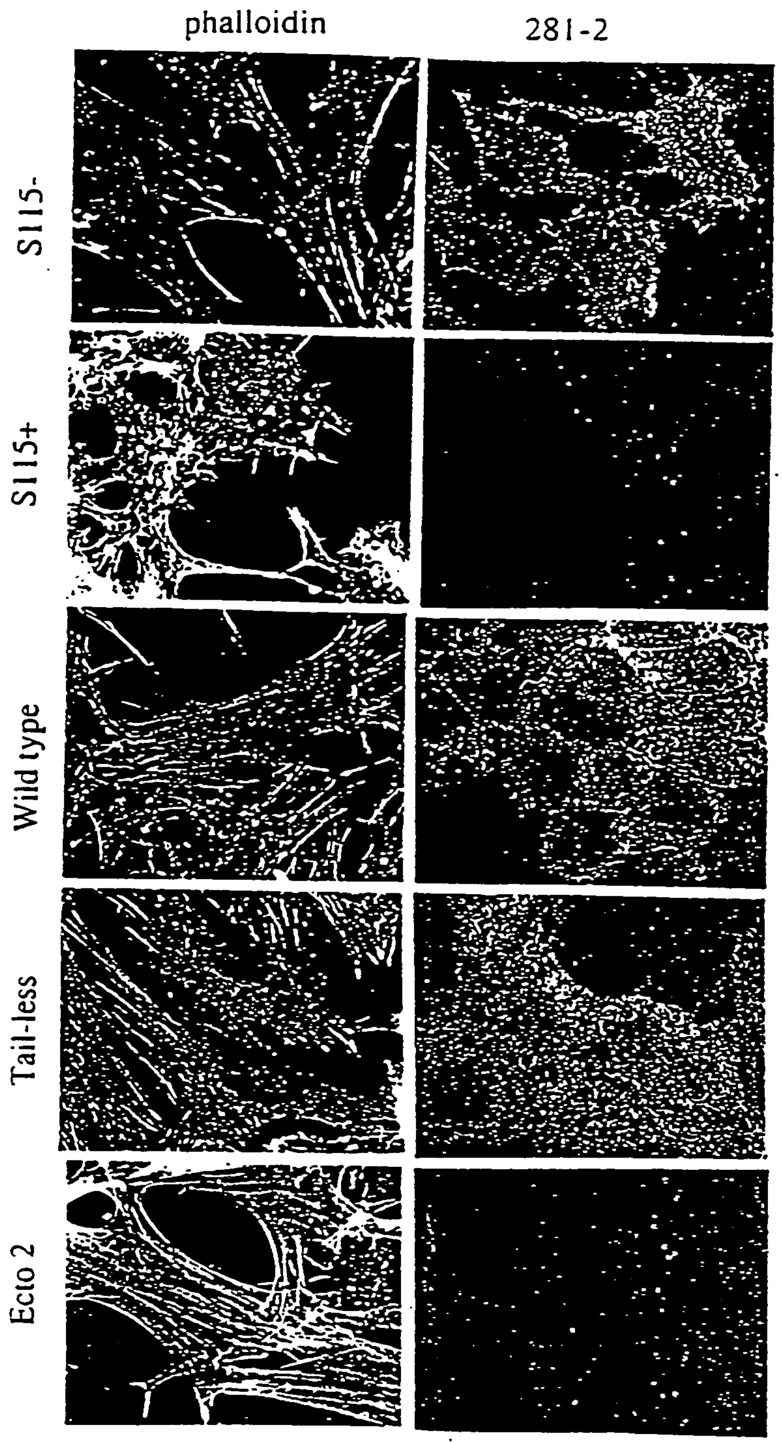
- FIGURE 3 -



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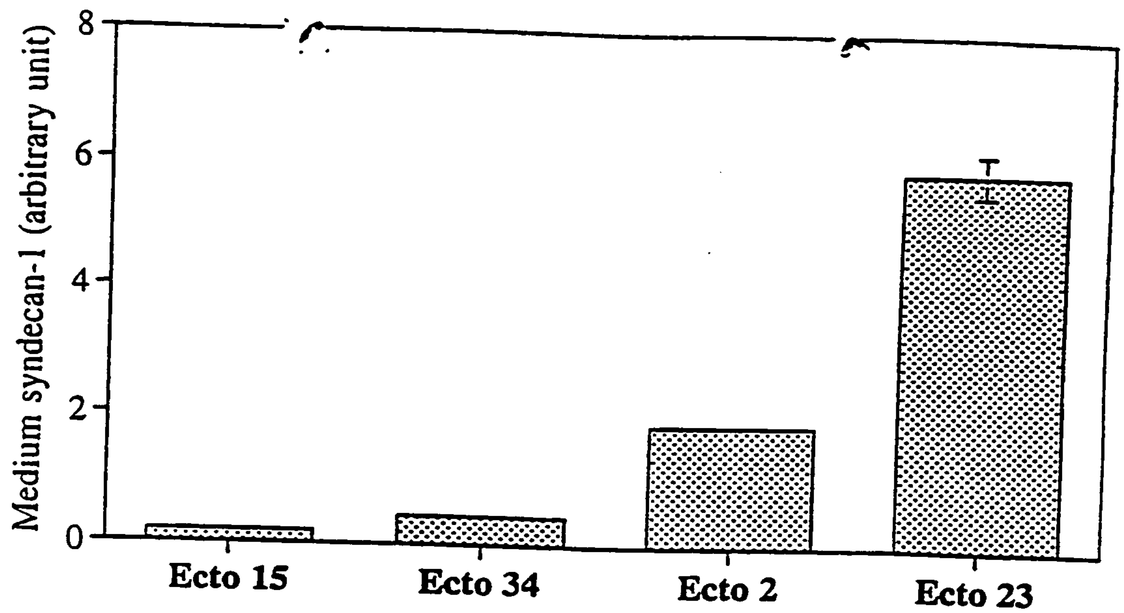
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6
4
2
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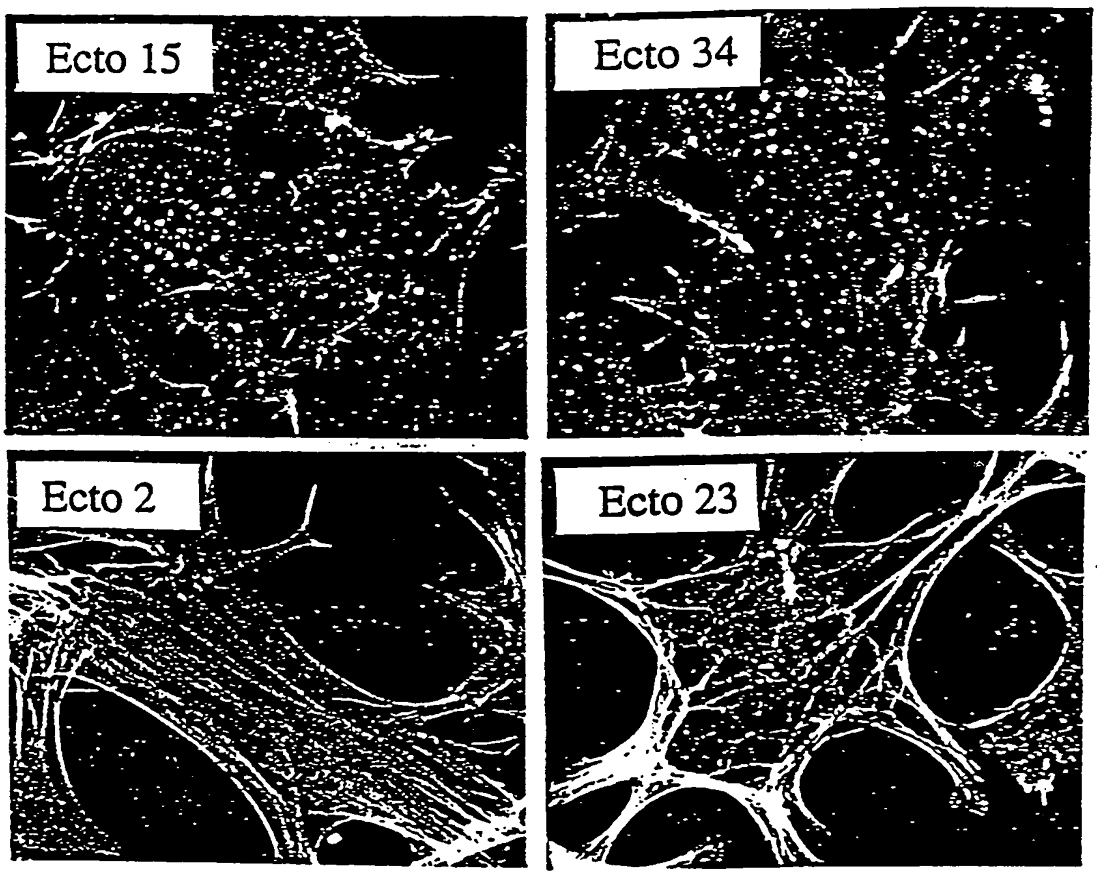
Ecto 34
Ecto 2
Ecto 23

- FIGURE 5 -



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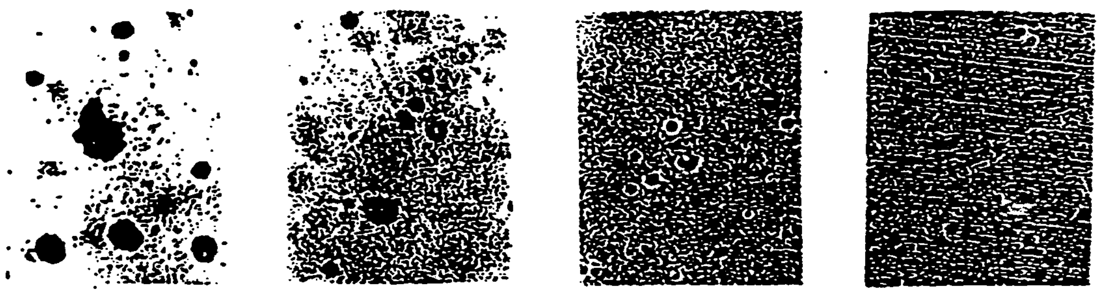
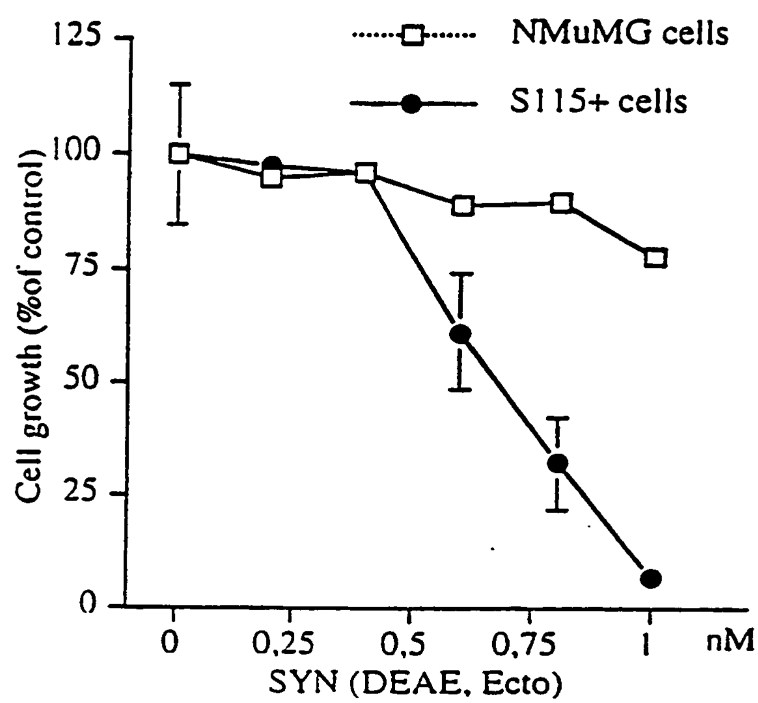


Ecto 15 Ecto 34 Ecto 2 Ecto 23

- FIGURE 7 -


NMuMG cells
S115+ cells
Cell growth (% of control)
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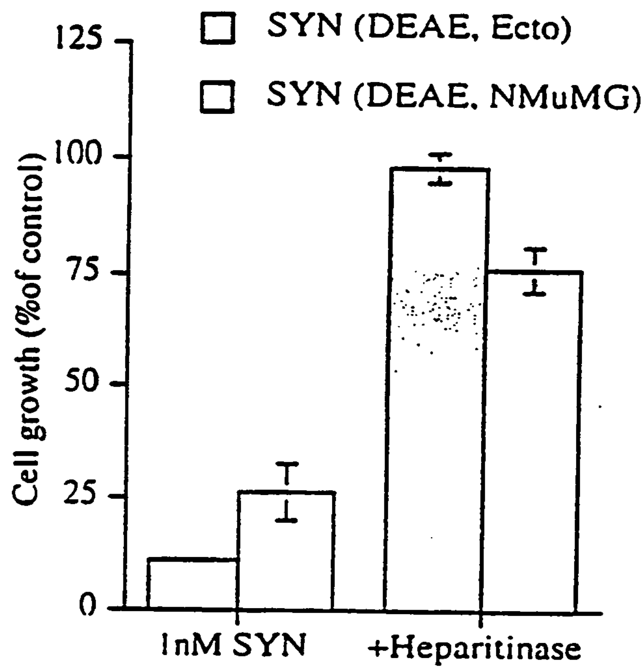
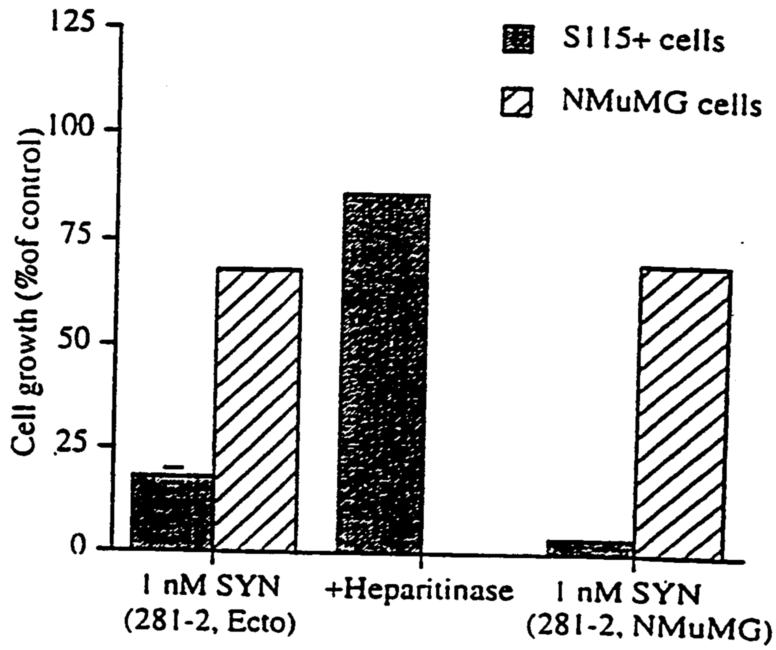
100
75
50
25
0
0
0,25
0,5
0,75
1
nM
SYN (DEAE, Ecto)

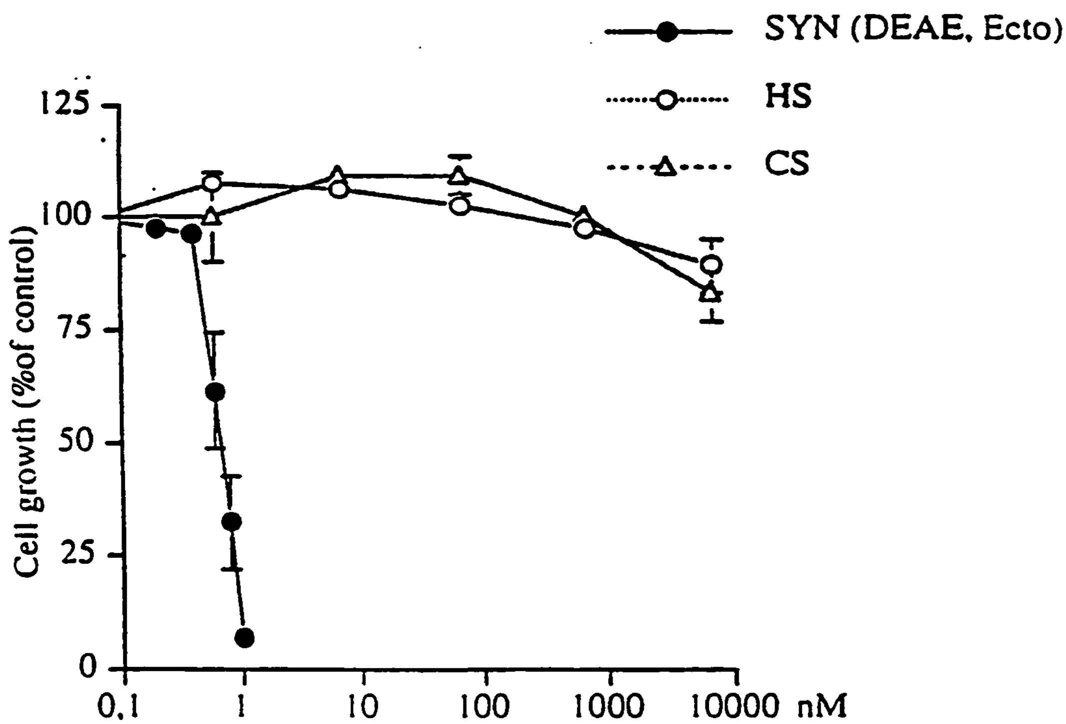
- FIGURE 8 -


SYN (DEAE, Ecto)
SYN (DEAE, NMuMG)
Cell growth (%of control)
125
100
75
50
25
0
1nM SYN
+Heparitinase

- FIGURE 9 -


125
100
75
50
25
0
Cell growth (%of control)
S115+ cells
NMuMG cells
1 nM SYN
(281-2, Ecto)
+Heparitinase
1 nM SYN
(281-2, NMuMG)

- FIGURE 10 -


SYN (DEAE, Ecto)
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CS
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100
75
50
25
0
Cell growth (%of control)
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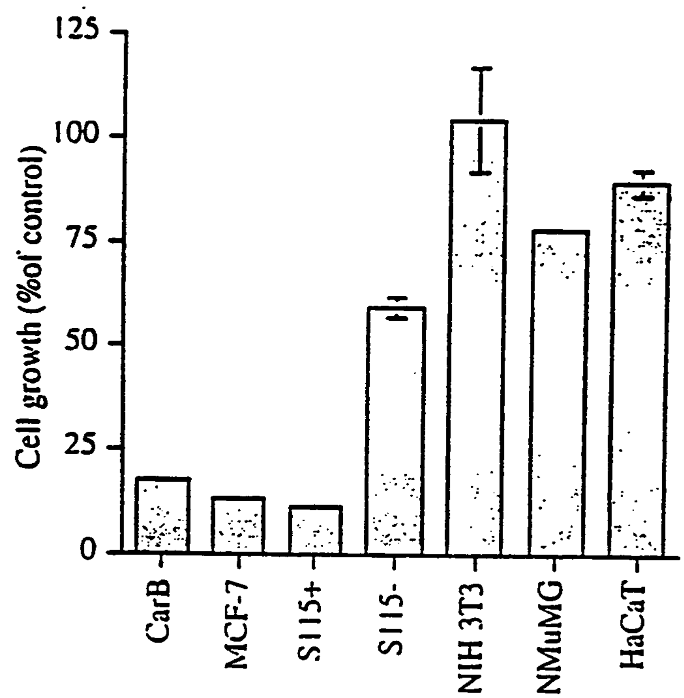
1
10
100
1000
10000 nM

- FIGURE 11 -


Cell growth (%of control)
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100
75
50
25
0
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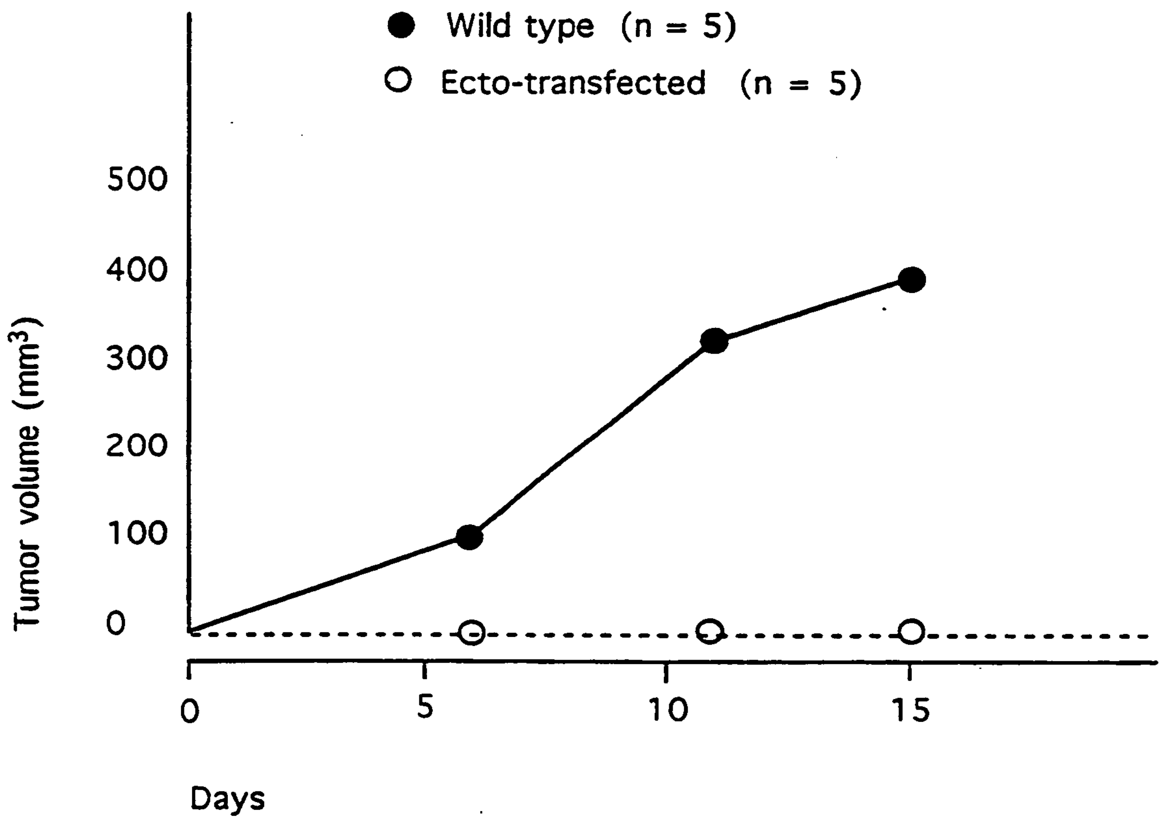
MCF-7
S115+
S115-
NIH 3T3
NMuMG
HaCaT

- FIGURE 12 -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Ecto-transfected (n = 5)
Tumor volume (mm³)
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400
300
200
100
0
0
5
10
15
Days

FIGURE 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI 95/00344

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 12162 (WÄRRI, ANNI, MAIJA ET AL.) 9 June 1994 cited in the application see page 8, line 5 - page 13, line 13	1-22
X,P	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.269, no.45, 11 November 1994, BALTIMORE, MD US pages 27795 - 27798 MARKKU MALI ET AL. 'SUPPRESSION OF TUMOR CELL GROWTH BY SYNDECAN-1 ECTODOMAIN.' see the whole document	1-22
X,P	WO,A,95 00633 (CHILDREN'S MEDICAL CENTER CORPORATION ET AL.) 5 January 1995 see page 37, line 17 - page 41, line 24	1-22

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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1

Date of the actual completion of the international search	Date of mailing of the international search report
20 November 1995	24.11.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Rempp, G

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-18
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-18 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/FI 95/00344

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9412162	09-06-94	AU-B- 5564994	22-06-94
		CA-A- 2150714	09-06-94
		EP-A- 0671909	20-09-95
WO-A-9500633	05-01-95	AU-B- 7112994	17-01-95

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